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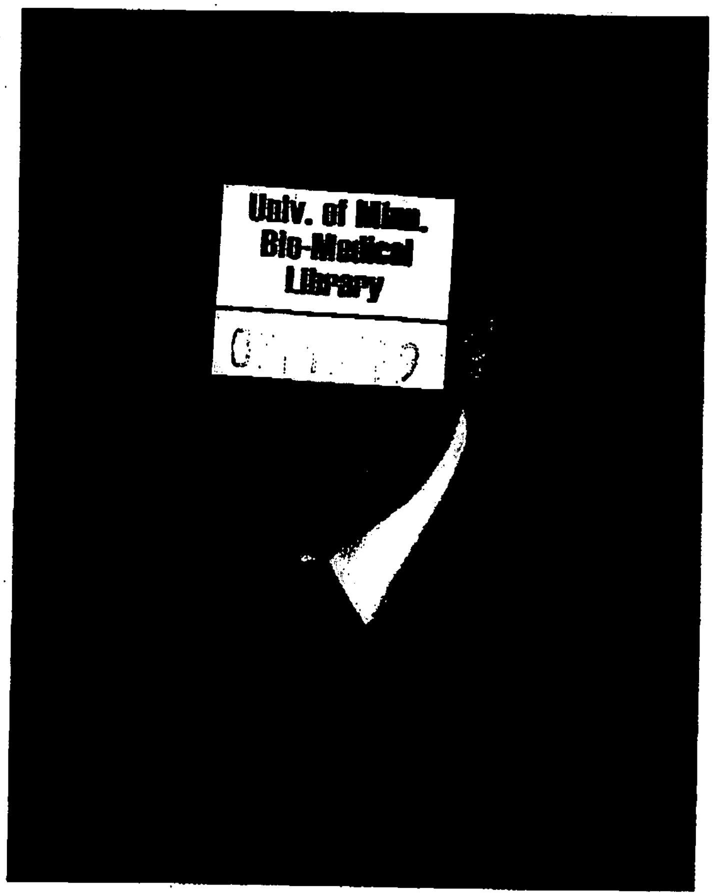
International Journal of Oncology

ISSN 1019-6439

An international journal devoted to Oncology Research and Cancer Treatment

VOLUME 15, NUMBER 2, AUGUST 1999



International Journal of Oncology

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Publication data: The International Journal of Oncology is published monthly. Author index and contents can be found at the end of each volume.

Bibliographic services: Articles in the International Journal of Oncology are listed in bibliographic services, including Current Contents/Life Sciencies, Science Citation Index, Scisearch, Research Alert, Index Medicus/MEDLINE, BIOSIS, Chemical Abstracts Service, Cambridge Scientific Abstracts, INIST/PASCAL, Sheffield University Biomedical Information Service and Current Awareness in Biological Sciences.

Annual subscription rates: Institutional subscription for 1999 (12 issues; including airmail postage): Europe US$630; (or equivalent in any other currency) rest of the world US$680 per year. Personal subscription Europe US$300; rest of the world US$350. Individual issues may be purchased at US$60. Checks should be made payable to D.A. Spandidos.

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Lack of elevated MAP kinase (Erk) activity in pancreatic carcinomas despite oncogenic K-*ras* expression

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Received April 26, 1999; Accepted May 17, 1999

Abstract. Activating mutations within the K-*ras* gene have been found in up to 90% of pancreatic carcinomas. Although multiple Ras effector pathways have been identified, the Raf protein kinases which are upstream regulators of the mitogen-activated protein kinases (MAPK/Erk) are believed to be the primary mitogenic effectors. Constitutive upregulation of this pathway by oncogenic *ras* is thought to promote cellular transformation. To explore the biological effects of mutated K-*ras*, we analyzed the Ras signaling pathway in a panel of cell lines derived from human pancreatic carcinomas. We found that despite high levels of Ras-GTP in each cell line expressing mutant K-*ras*, elevated levels of active Erk1 and Erk2 were not detectable under conditions of exponential growth or serum-starvation. Depending upon the cell line, the block in Erk signaling was observed to occur at either the level of Raf or Erk. Increased levels of active Erk1 and Erk2 were detected in only 2 out of 10 normal tissue-matched primary pancreatic tumors with mutated K-*ras*. Our results suggest that Erk signaling is not aberrantly upregulated in pancreatic cancers containing oncogenic K-*ras* mutations. The lack of Erk activation observed in both cell lines and primary tumor tissue suggests that constitutive Erk activation may not be required for tumor maintenance or progression in K-*ras* transformed pancreatic cells. We hypothesize that other Ras-dependent signaling pathways or an unidentified Raf/Mek-dependent pathway may be important for carcinogenesis in the pancreas. These findings may have important implications for drug treatment strategies which currently target the MAP kinase branch of the Ras signaling pathway.

Introduction

Mutations in *ras* genes occur in 20 to 30% of all human tumors (1,2). The *ras* gene family consists of H-, K-, and N-*ras* which encode closely related proteins with molecular weights of approximately 21,000 kDa. One of the *ras* family members, K-*ras*, is found mutated in approximately 90% of exocrine pancreatic cancer cases (3-6). These mutations occur in codons 12, 13 or 61, resulting in a decrease in the intrinsic and GAP-stimulated GTPase activity, thus rendering the Ras protein constitutively active in the GTP-bound form. The high incidence of K-*ras* mutations in pancreatic carcinomas suggests that oncogenic K-*ras* plays a critical role during pancreatic oncogenesis. Additional evidence implicating Ras in pancreatic oncogenesis includes the generation of mutated K-*ras* in N-nitroso-bis(2-oxopropyl)amine-induced pancreatic tumors in hamsters (7). Furthermore, the expression of antisense K-*ras* RNA in the AsPC-1, PaCa-2, PANC-1, and PSN-1 pancreatic cancer cell lines which have mutant K-*ras* alleles inhibited K-Ras protein production as well as the growth of these cells *in vitro*; tumorigenicity of the AsPC-1 and PaCa-2 cells *in vivo* was also suppressed (8,9).

Participants in the Ras signaling cascade have been elucidated, revealing a linear sequence of protein kinase activation (10,11). In response to an extracellular signal, Ras is activated via conversion from the inactive GDP-bound form to the active GTP-bound form (12,13). GTP-bound Ras recruits Raf protein kinase to the plasma membrane, resulting in its activation (14-16). Active Raf subsequently phosphorylates and stimulates the mitogen-activated protein kinase kinases, Mek1 and Mek2 (17,18). Mek in turn phosphorylates and activates the p44 and p42 mitogen-activated protein kinases (MAPK), also known as the extracellular signal-regulated kinases (Erk1 and Erk2) (19,20). Erk1 and Erk2 are believed to be the primary mediators of the proliferative signal generated by Ras, phosphorylating transcription factors and potential enzymatic effectors such as Jun, Elk-1, STAT, Myc, PHAS-1, $p90^{rsk}$ and PLA_2. Ultimately, this activation sequence results in the induction of immediate-early gene expression, such as c-*fos*, and is manifested externally by alterations in cell proliferation and/or differentiation (21).

There is increasing evidence that Ras may also mediate its effects through alternative downstream effector molecules other than Raf and MAPK. Recently, two H-Ras effector mutants incapable of binding or activating Raf were reported to result in the transformation of NIH 3T3 UNC fibroblasts (22). These mutants transformed cells synergistically when either

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Key words: pancreatic cancer, Ras, Erk

was co-expressed with activated Raf-1, suggesting that both Raf-dependent and Raf-independent signals contribute to Ras-mediated transformation. There is also accumulating evidence that different targets are required for Ras-mediated transformation depending upon the cell type. For example, although constitutively active Ras and Raf mutants each induced transformation of NIH 3T3 fibroblasts, only mutant Ras was able to transform RIE-1 epithelial cells despite MAPK activation by either mutant Ras or Raf in these cells. These observations suggest that Raf-independent pathways are required for transformation by oncogenic Ras in epithelial cells (23).

Conclusions regarding Ras transformation and signaling have mainly been reached by overexpressing Ras signaling proteins in rodent fibroblasts. However, the majority of human cancers associated with mutated Ras, including pancreatic carcinomas, are epithelial in origin. It is possible that distinct differences in Ras-mediated transformation and signaling may exist between *in vitro* fibroblast models and epithelial cell-derived tumors. To explore the consequences of mutated K-*ras* in pancreatic carcinomas, we biochemically characterized the activation state of the Ras/MAPK signal transduction cascade in a panel of eight human pancreatic cancer cell lines. We demonstrate that in only four of the seven cell lines with oncogenic K-*ras*, Raf and Mek activities were elevated. However, no significant increase in active Erk was detectable under conditions of exponential growth or serum starvation, suggesting downregulation of the Erk pathway. In the other three cell lines with oncogenic K-*ras*, Raf/Mek/Erk were not significantly activated despite measurable Ras activation. This observation suggests that in some cases, the initial signal fails to be transmitted downstream possibly due to downregulation of protein kinases and/or diversion of the signal to an alternative mitogenic pathway. A similar lack of Erk activation was also apparent in pancreatic tumor tissue compared to matched normal tissue. These findings demonstrate that the presence of oncogenic K-*ras* does not necessarily result in continuous Erk activation in pancreatic carcinomas, contrary to predictions based upon fibroblast expression systems.

Materials and methods

Cell culture. The human pancreatic cancer cell lines and the normal human fibroblast cell line WI-38 were obtained from the ATCC (Rockville, MD) and cultured as recommended. The AsPC-1, Capan-1, Capan-2, HPAF-II, PANC-1 and PaCa-2 cell lines have been previously reported to contain an activating mutation at codon 12 within the K-*ras* gene; whereas the cell line BxPC-3 expresses wild-type K-*ras* (9,24). Rat-1 fibroblasts and the Rat1-H*ras*[val12] cell line were generated and cultured as described previously (25). The cell line 833K used as a control, was derived from a testicular germ cell tumor (Dr Nicholas Vogelzang, University of Chicago, IL); genomic DNA was isolated from this cell line and the presence of wild-type H-, K- and N-*ras* was confirmed by sequencing (Yip-Schneider M, data not shown). For all experiments, exponentially growing cells were harvested between 50-80% confluency. Cells between 50-80% confluency were serum-starved overnight followed by stimulation with 10% fetal calf serum (FCS) for the indicated lengths of time.

Ras nucleotide loading. Growing cells were incubated in phosphate-free media containing 10% dialyzed FCS for 2 h. Cells were then metabolically labeled with orthophosphate (200 µCi/ml) for 3 h followed by lysis in buffer containing 25 mM Tris pH 7.5, 150 mM NaCl, 16 mM $MgCl_2$, 1% NP-40, 1 mM PMSF, 20 µg/ml aprotinin, 50 µg/ml leupeptin, and 5 µg/ml Ras antibody (Y259, Oncogene Science) or Rat IgG as the negative control. Cells were scraped, aspirated through a 25G needle and centrifuged for 10 min at 14,000 x g at 4°C. After adjusting to 0.5 M NaCl, the lysates were mixed by rotation at 4°C for one hour. Equivalent amounts of total protein were then incubated with Gamma Bind G Plus beads for an additional hour followed by washes with lysis buffer lacking antibody. Elution was performed in 20 µl of elution buffer (2 mM EDTA, 0.2% SDS, and 2 mM DTT) at 100°C for 3 min followed by centrifugation at 14,000 x g for 5 min. Supernatants were stored at -80°C. Samples were spotted along with GDP/GTP standards onto polyethyleneimine cellulose plates coated with fluorescent indicator and chromatographically separated in 1 M KH_2PO_4 pH 3.5 buffer (26). Labelled nucleotides were visualized by autoradiography and quantitated by densitometry.

Raf kinase assay. The Raf kinase assay was performed basically as described previously with some modifications (27). Following cell lysis, equivalent amounts of total protein were immunoprecipitated with a Raf-1 antibody (C-12, Santa Cruz Biotechnology Inc.) for 2 h at 4°C. Complexes were collected by incubation with Protein A Sepharose CL4B for an additional hour. The washed complexes were then incubated with GST-Mek and GST-Erk. These reactions were subsequently incubated with myelin basic protein (MBP) and [^{32}P]-ATP. The reaction mixture was spotted onto P81 filters, washed and quantitated by scintillation counting. Equivalent amounts of immunoprecipitated Raf-1 present in the complexes were confirmed by Western blot analysis.

Mek kinase assay. Assays performed as described above for Raf except immunoprecipitations were performed with anti-Mek1/Mek2 antibody (Transduction Laboratories), and the complexes were assayed by the addition of GST-Erk.

Immunoblot analysis was performed by lysing cells in RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 20 µg/ml aprotinin, 1 mM Na_3VO_4). Proteins were resolved by SDS-PAGE on 10% or 4-20% gradient gels (Novex) and transferred to Immobilon P membranes (MSI). The blots were probed with either a phospho-specific (Ser217/221) Mek1/Mek2 antibody (New England Biolabs) or the Mek1/Mek2 antibody (Transduction Laboratories) according to the manufacturer's protocol followed by ECL (Amersham) to detect phosphorylated Mek1/Mek2 and total Mek protein levels respectively.

MAP kinase activation. Cell lysates were prepared as described above and analyzed by immunoblot analysis with either a phospho-specific (Thr202/Tyr204) Erk1/Erk2 antibody (New England Biolabs) or an Erk1 antibody (SC-94, Santa Cruz

Table I. Cell lines expressing wild-type or oncogenic Ras used in this study.

Cell line	K-ras[a]	Pathology and source of tumor/cell line
Rat-1	-	Untransformed, immortalized rat fibroblasts
Rat1-H*ras*[val12]	+ (H-*ras*)	Rat fibroblasts transformed by H-*ras* mutated at codon 12
WI-38	-	Normal, diploid human fetal lung fibroblasts
833K	-	Human testicular germ cell tumor
BxPC-3	-	Primary human pancreatic adenocarcinoma
Hs766T	+[b]	Human pancreatic carcinoma from a lymph node metastasis
AsPC-1	+	Primary human pancreatic adenocarcinoma from peritoneal ascitic fluid
Capan-1	+	Human pancreatic adenocarcinoma from a liver metastasis
Capan-2	+	Primary human pancreatic adenocarcinoma
HPAF-II	+	Primary human pancreatic adenocarcinoma from peritoneal ascitic fluid
MIA PaCa-2	+	Primary human pancreatic carcinoma
PANC-1	+	Primary human pancreatic epithelioid carcinoma

[a]The presence or absence of an activating mutation within the K-*ras* gene is indicated by '+' or '-' respectively. [b]The Hs766T cell line was previously reported to possess wild-type K-*ras*, but upon sequencing exon 2 of the K-*ras* gene, we discovered an activating mutation at codon 61.

Biotechnology) followed by ECL (Amersham) to detect phosphorylated Erk1/Erk2 and total Erk protein levels respectively.

Patient tissue samples. Eleven matched pancreatic adenocarcinomas and normal adjacent tissues were frozen in liquid nitrogen following surgical removal and stored at -80°C. The frozen tissue was briefly homogenized in RIPA lysis buffer described above with additional inhibitors (20 mM ß-glycerophosphate, 1 mM sodium fluoride, pepstatin (1 µg/ml) and leupeptin (1 µg/ml). Lysates were clarified by centrifugation at 13,000 x g for 10 min, followed by boiling in sample buffer. Protein concentrations were determined using the BCA protein assay kit (Pierce). Equivalent amounts of total protein were analyzed by immunoblot analysis.

K-ras mutation analysis. Genomic DNA was prepared by incubating the tissue in lysis buffer (50 mM Tris pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K) overnight at 55°C. RNAse (0.1 mg/ml) was added and the incubation was continued for 2 h at 37°C. The sample was then extracted with phenol, phenol:chloroform, and ethanol precipitated. K-*ras* exon 1 of the resuspended genomic DNA (0.5 µg) was amplified by PCR (5' primer = 5'-ATGACTGAATATAAACTTGT-3'; 3' primer = 5'-CTCTATTGTTGGATCATATT-3') (28). K-*ras* mutation specific oligonucleotides (Oncogene) were utilized to detect mutations at K-*ras* codon 12 in the PCR-amplified products by dot blot hybridization (29).

Results

To explore the biological consequences of mutated K-*ras* in pancreatic cancer, we biochemically characterized the Ras signal transduction pathway in a panel of eight exocrine pancreatic carcinoma cell lines established from human pancreatic tumor tissue (Table I). Primary human WI-38 diploid cells and untransformed or H-*ras*[val12] transformed Rat-1 fibroblasts were analyzed in parallel as negative and positive controls respectively. The 833K germ cell tumor cell line was examined as a type of cancer independent of oncogenic *ras*. The percentage of Ras bound to GTP, reflecting total Ras protein activation, was found to be elevated significantly between 21% and 33% in the AsPC-1, HPAF-II, Capan-1, Capan-2, PANC-1, and PaCa-2 pancreatic cancer cell lines and up to 52% in the Rat1-H-*ras*[val12] cells, consistent with the presence of activating *ras* mutations in these cell lines (Fig. 1). Surprisingly, the HS766T pancreatic cancer cell line reportedly containing wild-type K-*ras* alleles exhibited 21% GTP-bound Ras. We subsequently isolated genomic DNA from this cell line, sequenced exons 1 and 2 of the K-*ras* gene, and discovered the presence of an activating mutation at codon 61 in one allele (CAA to CAC, Yip-Schneider M, data not shown). In contrast, the Rat-1 and WI-38 control cells as well as the BxPC-3 pancreatic and 833K germ cell tumor cell lines displayed only 2-7% GTP-bound Ras consistent with the low level of active Ras present in growing cells expressing wild-type Ras.

Ras activation is generally thought to upregulate the Raf, Mek and Erk protein kinase cascade. We therefore

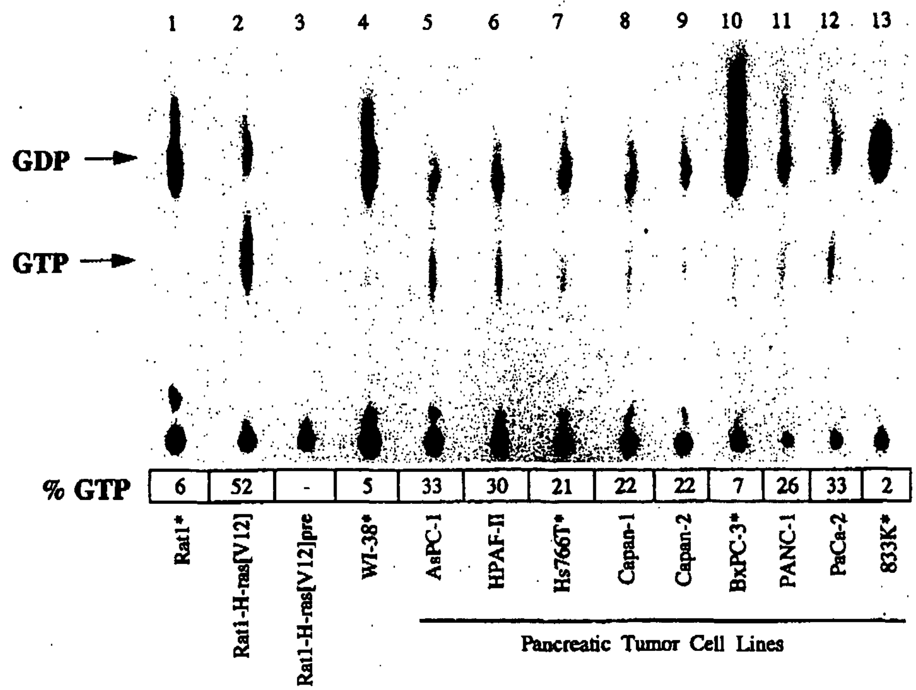
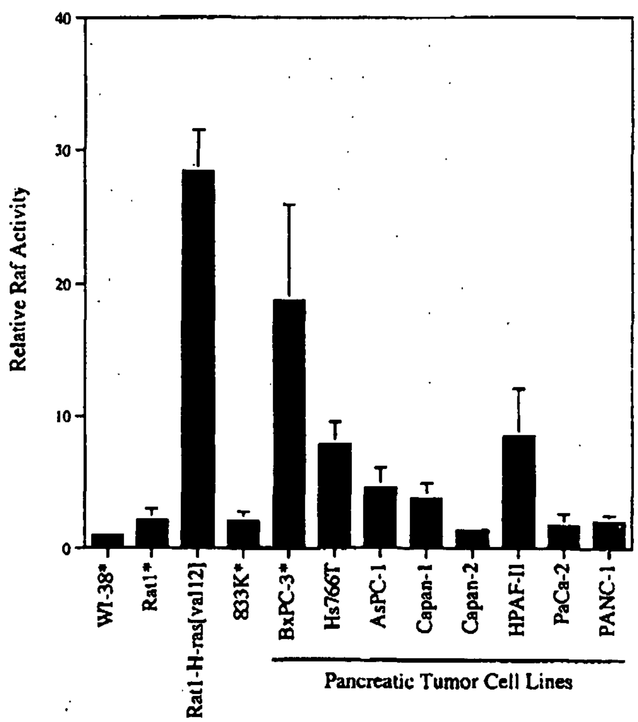


Figure 1. Ras activation state in pancreatic carcinoma cell lines. Radiolabelled nucleotides bound to Ras immunoprecipitated from growing pancreatic tumor cell lines (lanes 5-12), control lines (Rat-1, Rat1-H-*ras*[val12], WI-38; lanes 1, 2, and 4), or the 833K germ cell tumor cell line (lane 13) were extracted, chromatographically separated, and visualized by autoradiography. As a negative control, the Rat1-H-*ras*[val12] lysate was immunoprecipitated with Rat IgG and treated identically (lane 3). Migrations of GDP and GTP are indicated by the arrows. Cell lines expressing wild-type K-*ras* are indicated by the asterisk. Quantitation of %GTP for each cell line was performed by densitometry and adjusted for phosphate number. Results were similar in two separate experiments.



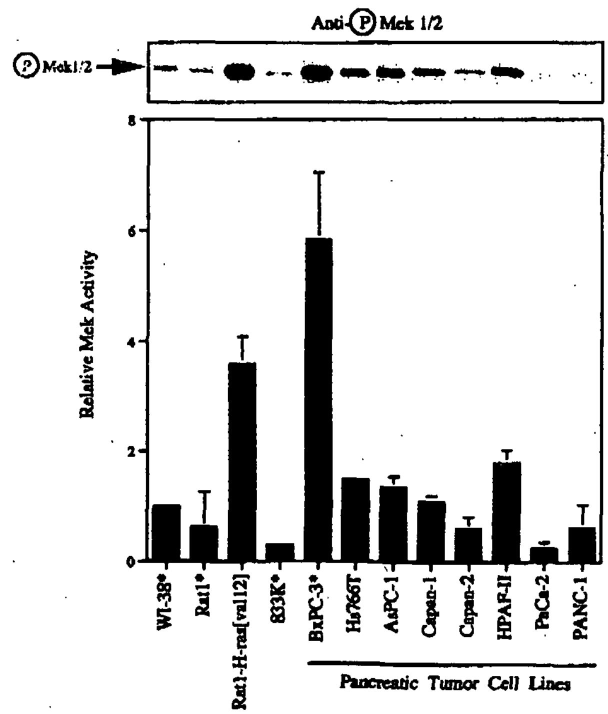
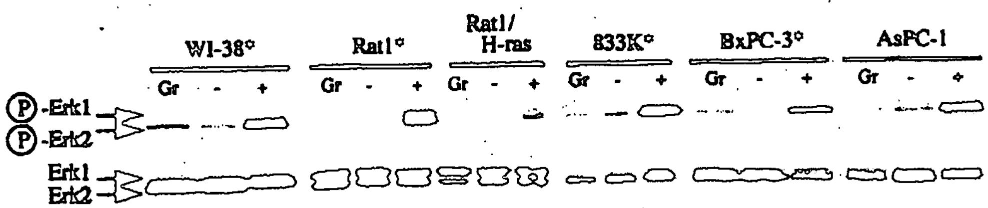
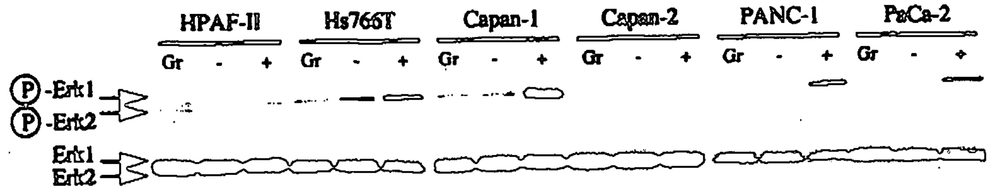


Figure 2. Raf-1 activity in pancreatic carcinoma cell lines. Raf-1 was immunoprecipitated from the pancreatic tumor cell lines, control lines (WI-38, Rat-1, Rat1-H-*ras*[val12]) as well as the 833K germ cell tumor cell line. Raf-1 activity in the immunoprecipitates was quantitated by a coupled *in vitro* kinase assay using MBP as the substrate. Relative Raf-1 activity for each cell line was determined based upon the level of Raf-1 activity in WI-38 cells (set equal to 1) and expressed as the mean ± SD from three independent experiments. Cell lines expressing wild-type K-*ras* are

Figure 3. Mek activation in pancreatic cell lines. Pancreatic carcinoma and control cell lines were assayed for Mek1/Mek2 activity using a coupled *in vitro* kinase assay. Relative Mek activity was determined based upon the level of Mek activity in WI-38 cells (set equal to 1) and expressed as the mean ± SD from two independent experiments. Wild-type K-*ras* expressing cell lines are indicated by the asterisk. A phospho-Mek1/2 specific antibody was also used to determine the level of active Mek in total cell lysates by immunoblot analysis. Detection of active, phosphorylated Mek is indicated



	WI-38* Gr	WI-38* -	WI-38* +	Rat1* Gr	Rat1* -	Rat1* +	Rat1/H-ras Gr	Rat1/H-ras -	Rat1/H-ras +	833K* Gr	833K* -	833K* +	BxPC-3* Gr	BxPC-3* -	BxPC-3* +	AsPC-1 Gr	AsPC-1 -	AsPC-1 +
Relative Active Erk	1.0	0.6	10	0.2	0.4	5.0	0.3	0.7	1.9	0.4	0.9	9.0	1.0	0.4	4.3	1.3	1.1	5.9
Fold Induction	-	-	16.7	-	-	12	-	-	2.7	-	-	10	-	-	10.7	-	-	5.4



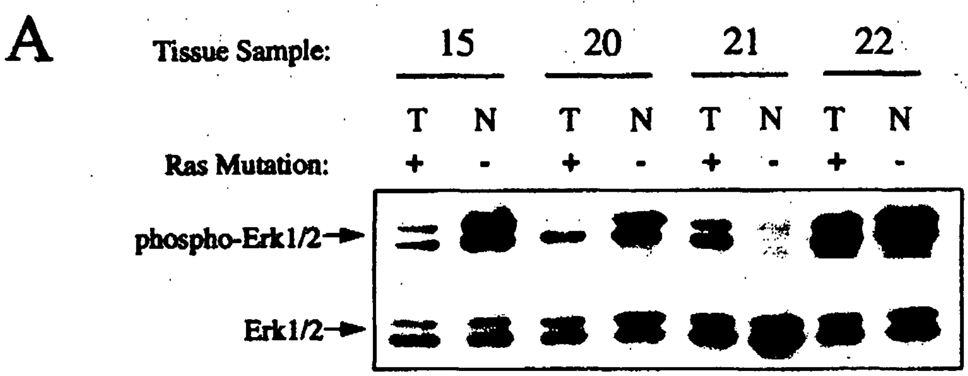
	HPAF-II Gr	HPAF-II -	HPAF-II +	Hs766T Gr	Hs766T -	Hs766T +	Capan-1 Gr	Capan-1 -	Capan-1 +	Capan-2 Gr	Capan-2 -	Capan-2 +	PANC-1 Gr	PANC-1 -	PANC-1 +	PaCa-2 Gr	PaCa-2 -	PaCa-2 +
Relative Active Erk	1.0	0.6	1.1	0.8	1.3	3.4	0.8	0.8	3.3	0.1	0.1	0.2	0.4	0.4	4.3	0.2	0.3	1.9
Fold Induction	-	-	1.8	-	-	2.6	-	-	4.1	-	-	2.0	-	-	10.7	-	-	6.3

Figure 4. MAP kinase activation in pancreatic cancer cells. MAPK/Erk activation was determined in growing (Gr), serum-starved (-) or FCS-stimulated (+) pancreatic cancer cells (BxPC-3, AsPC-1, HPAF-II, Hs766T, Capan-1, Capan-2, PANC-1, PaCa-2), the control lines (WI-38, Rat-1, Rat1-H-*ras*[val12]) or the 833K germ cell tumor cell line. Total cell lysates were prepared from each cell line and activated phospho-Erk1/Erk2 was detected by immunoblot analysis employing a phospho-specific Erk1/Erk2 antibody as indicated. The blot was re-probed with anti-Erk antibody to control for loading. Cell lines expressing wild-type K-*ras* are indicated by the asterisk. Following band quantitation by densitometry and normalization to total Erk protein, relative expression of active Erk was calculated compared to WI-38 cells (set equal to 1). Fold induction corresponds to the level of induction of active Erk upon serum stimulation of starved cells. These results are representative of two separate experiments.

quantitated Raf-1 activity in the pancreatic carcinoma and control cell lines utilizing a coupled *in vitro* kinase assay (Fig. 2). The results are expressed relative to the level of Raf-1 activity measured in primary WI-38 cells. Two- to three-fold increases in Raf-1 activity were detected in two control cell lines without oncogenic Ras, the 833K germ cell tumor line and the immortalized Rat-1 fibroblasts, suggesting that slight increases in Raf-1 activity may correlate with cell immortalization/transformation regardless of the presence of oncogenic Ras. Of the seven *ras* mutant pancreatic carcinoma cell lines which had elevated GTP-bound Ras (AsPC-1, HPAF-II, Hs766T, Capan-1, Capan-2, PANC-1 and PaCa-2), four- to nine-fold increases in Raf-1 activity were detected in only four of the cell lines (AsPC-1, HPAF-II, Hs766T, Capan-1). In contrast, Raf-1 activity in the other three pancreatic cell lines with high GTP-bound Ras (Capan-2, PaCa-2, PANC-1) was low, comparable to the level measured in the untransformed control. The wild-type K-*ras* BxPC-3 pancreatic cell line, which exhibited only basal levels of GTP-bound Ras, exhibited unexpectedly high Raf-1 activity, similar to that detected in the H-*ras*[val12]-transformed Rat-1 fibroblasts.

To confirm the Raf activity results, Mek activity was measured in exponentially growing pancreatic carcinoma and control cell lines using a coupled *in vitro* kinase assay (Fig. 3). Mek1/Mek2 activity was elevated four- and six-fold respectively in the H-*ras*-transformed Rat-1 and BxPC-3 cell lines relative to that measured in the untransformed Rat-1 and WI-38 control cells. The high level of Mek1/Mek2 activity correlated with the high level of Raf-1 activity detectable in these two cell lines. Slight increases in Mek activity, approximately 1.5-fold, were also consistently detected in the Hs766T, AsPC-1, Capan-1 and HPAF-II pancreatic cell lines, which also correlated with the increased Raf-1 activity measured in these cell lines. Lower levels of Mek activity were found in the Capan-2, PANC-1, and PaCa-2 pancreatic carcinoma cells as well as in the 833K germ cell tumor cell line, consistent with the low level of Raf-1 activation observed in these cells. These results were also confirmed using a phospho-Mek specific antibody to detect active, phosphorylated forms of Mek1/Mek2 in total cell lysates by immunoblot analysis (Fig. 3). In general, the level of active Mek reflected the pattern of Raf-1 activity in the cell lines.

The activation state of p44/p42 MAP kinases (Erk1/Erk2), downstream targets of Mek, was measured in each of the pancreatic carcinoma cell lines and compared to the control cell lines. Total cell lysates were prepared from exponentially growing cells, serum-starved cells or serum-starved cells stimulated with FCS. Cell lysates were then assayed by immunoblot analysis using a phospho-Erk specific antibody to detect the active, phosphorylated forms of Erk1/Erk2 (Fig. 4) (30,31). Surprisingly, the level of Erk phosphorylation/activation in all growing cell lines regardless of *ras* mutation status was equal to or less than that detected in WI38 cells, which have very low levels of active Raf and Mek. This basal level of Erk phosphorylation presumably reflects the small fraction of cells actively proliferating at any one time



B

tumor sample	4	7	11	15	16	17	18	20	21	22
Erk activation	+	-	-	-	-	-	-	-	+	-
K-Ras mutation (codon 12)	GAT (Asp)	ND	ND	GAT (Asp)	GAT (Asp)	GTT (Val)	GAT (Asp)	CGT(Arg) TGT(Cys)	CGT(Arg) TGT(Cys)	GAT (Asp)

Figure 5. Lack of Erk activation in primary pancreatic tumors. A, A representative immunoblot of lysates prepared from pancreatic tumor tissue (T) and matched normal, adjacent tissue (N) obtained from 4 patients is shown. The presence (+) or absence (-) of K-*ras* mutation, and expression of phospho-Erk1/2 and total Erk1/2 are indicated. B, Relative Erk activation in primary pancreatic tumor tissue versus matched normal, adjacent tissue from 10 patients was analyzed by immunoblotting with the phospho-specific Erk antibody and summarized in the table. Elevated levels of active Erk in tumor versus normal tissue is indicated by (+). Lack of Erk activation in tumor tissue is indicated by (-). Ras mutation status of the pancreatic tumor tissue was determined by PCR amplification of K-ras exon 1 from genomic DNA followed by allele specific hybridization. The sequence at codon 12 of wild-type K-*ras* is GGT (GLY). ND, not determined.

in culture. Low levels of Erk phosphorylation were also detected in serum-starved cells. Upon stimulation of serum-starved cells with FCS for 5 min, an increase in active, phosphorylated Erk1/Erk2 occurred in most of the pancreatic carcinoma and control cell lines, demonstrating functional MAP kinase signaling in response to normal mitogenic stimuli. Similar results were obtained in some of the pancreatic cancer cell lines upon stimulation with EGF (Yip-Schneider M, data not shown). However, the serum-induced activation of Erk was attenuated in several of the pancreatic cell lines as well as in the H-*ras*-transformed Rat-1 cells. Nearly complete abrogation of serum-induced Erk phosphorylation was observed in the Capan-2 and HPAF-II pancreatic cell lines. Taken together, these results demonstrate that upregulation of the MAPK pathway is not observed in any of the pancreatic tumor cell lines, even in the BxPC-3 cell line which displayed extremely high levels of activated Raf and Mek, or in the H-*ras*-transformed Rat-1 fibroblasts. In fact, serum-induced phosphorylation/activation of Erk was partially or strongly attenuated in several cases, raising the possibility that the Erk signaling pathway may be subject to downregulation in the presence of a constitutive Ras/Raf signal.

To determine whether the lack of Erk activation observed in the pancreatic cell lines was the result of *in vitro* culturing or was biologically significant, we measured the amount of active, phosphorylated Erk in ten matched sets of primary human pancreatic tumor and adjacent normal pancreatic tissue. Increased levels of active Erk1/Erk2 were detected in only 2 out of 10 pancreatic adenocarcinomas, despite the presence of mutated K-*ras* genes identified by PCR amplification of genomic DNA followed by allele-specific hybridization (Fig. 5A and B). There was no significant difference between the cancer tissues and the corresponding matched normal control tissues as determined by the non-parametric signed rank test (P=0.5). These results demonstrate that Erk1 and Erk2 are also not consistently activated in tissues extracted from humans with pancreatic adeno-carcinomas, confirming our *in vitro* results.

Discussion

Recent studies have suggested that mitogenic Ras signaling is more complex than simply a linear flow of information from Ras-GTP to Raf to Mek to MAPK/Erk. Not only have proteins other than Raf been shown to be directly regulated by Ras, but Raf itself has been shown to be mitogenic in the absence of Erk stimulation (22,23,32). Furthermore, fibroblast and epithelial cells transformed in culture by oncogenic *ras* require different Ras effectors (23). In light of these complexities, we chose to examine human pancreatic cancer as a model for a Ras-dependent cancer. With upwards of 90% of all exocrine pancreatic carcinomas containing

Table II. Summary table of relative activation of the Ras signaling pathway in pancreatic tumor and control cell lines.[a]

Cell line	K-ras mutation	%GTP	Raf-1	Mek1/2	Erk1/2
WI-38	-	-	-	-	-
Rat1	-	-	-	-	-
Rat1-Hras	+ (H-*ras*)	++	++	+	-
833K	-	-	-	-	-
BxPC-3	-	-	++	+	-
Hs766T	+	+	+	+/-	-
AsPC-1	+	+	+/-	+/-	-
Capan-1	+	+	+/-	+/-	-
Capan-2	+	+	-	-	-
HPAF-II	+	+	+	+/-	-
PaCa-2	+	+	-	-	-
PANC-1	+	+	-	-	-

[a]Relative activation of Ras, Raf-1, MEK1/2, and Erk1/2 in exponentially growing cells is expressed as -, +/-, +, or ++ corresponding respectively to negligible, slight, moderate, or high levels of activation based on the absolute numbers stated in the figures.

activating mutations in the K-*ras* gene, this tumor type should provide useful information on the physiologically relevant Ras function in human solid tumors.

Upon examining multiple human pancreatic tumor cell lines, we found similarities in the activation states of the Ras signaling components as well as differences, presumably reflecting the heterogeneity of the exocrine pancreatic carcinomas from which the cell lines were derived (summarized in Table II). First, we confirmed that the presence of a mutant K-*ras* allele does in fact result in a high level of intracellular Ras-GTP. The 21-33% Ras-GTP observed in these tumor cells is well above the 15-20% previously shown to be mitogenic in other defined cell systems (33). In four out of the seven mutant K-*ras* cell lines examined, Ras activation resulted in increased Raf-1 and Mek activities. However, in three of the pancreatic cell lines, active Raf-1 and Mek were difficult to detect despite significant Ras activation. A number of explanations can be provided which might explain the presence of transforming K-*ras* but the apparent lack of signaling between K-Ras/Raf/Mek which we observed in the Capan-2, PaCa-2 and PANC-1 pancreatic cancer cell lines. One possiblity is that oncogenic K-*ras* function in pancreatic cancer may be mediated by an effector pathway other than Raf, even though K-*ras* is an effective activator of Raf-1 (34-36). Other candidate Ras-dependent mitogenic effectors include PI-3-kinase and RalGDS (37-42). Alternatively, the lack of Raf/Erk activation may reflect intrinsic downregulation as a result of constitutive Ras activation. In fibroblasts, constitutive activation of the Ras pathway induces strong inhibitory responses. For example, in NIH 3T3 and Rat-1 cells stably transformed by oncogenic Ras or Raf, Erk1 and Erk2 are not constitutively active and instead appear to be less responsive to mitogenic growth factors (43). The specific activity of the RasGAP protein is also dramatically increased in Ras-transformed cells in what appears to be an ineffective effort by the cell to reduce the amount of Ras-GTP (Marshall M, personal communication). It is therefore possible that exocrine pancreatic cells selectively downregulate the Raf/MAPK pathway and channel growth signals through alternative mitogenic pathways. Finally, another possibility which must be considered is that very low levels of activation not readily detectable by our assays may be capable of contributing to pancreatic oncogenesis. For example, suppression of Raf or Erk activities in the presence of constitutive K-Ras GTP might be relieved during a restricted point in the cell cycle, resulting in a large but transient spike of kinase activity undetectable by our assays.

Erk activation was not increased above the basal levels normally observed in asynchronous cell populations in any of the pancreatic cell lines under conditions of exponential growth or serum-starvation, even in the wild-type K-*ras* BxPC-3 cell line or in the H-*ras* transformed Rat-1 fibroblasts, both of which exhibited extremely high Raf and Mek activities. Erk activation was also not consistently detected in primary pancreatic tumor tissue. Taken together, these data suggest that constitutively active Erk is not essential for maintenance of the transformed state in pancreatic carcinomas. Nevertheless, since most of the pancreatic cell lines tested are factor-dependent and demonstrate mitogen-inducible Erk phosphorylation, Erk function appears to be required for normal, homeostatic proliferation. In several of the cell lines, serum-induced Erk activation was attenuated suggesting the existence of negative regulatory mechanisms directed at repressing Erk as recently described in rodent fibroblast lines transformed by v-Src, v-Sis, v-Ras or v-Raf (43,44). We have evidence that downregulation of the Erk pathway in some of the pancreatic cancer cell lines may be mediated by a tyrosine or dual specificity phosphatase (Yip-Schneider M, unpublished observations). Supporting our findings in pancreatic carcinomas, a recent report demonstrated that in K-*ras* transformed human astrocytoma cells, Erk is not constitutively activated; furthermore, Erk activation in response to EGF is attenuated (45). Such negative regulatory mechanisms may be invoked by transformed cells to control the chronic stimulation of signaling pathways which may occur during oncogenic transformation.

The majority of pancreatic cancers are adenocarcinomas which are derived from epithelial cells. Utilizing pancreatic tumor cell lines as a model of pancreatic cancer, we have demonstrated activation of at least one Ras pathway intermediate in each of the cell lines analyzed. This observation was noted even in the cell line without a Ras mutation, suggesting that activation of the Ras pathway is important in the development of pancreatic cancer. However, the activation signal was not neccessarily transduced completely downstream and was found to be blocked or diverted downstream of either Ras or Mek. Moreover, in both the

pancreatic cell lines and human tumor tissue, Erk activity was not upregulated by oncogenic K-*ras*. The lack of high Erk activity in the cell lines, which appears to be mirrored in the human cancer specimens, suggests that the cell lines are a relevant surrogate in these studies. Taken together, our findings suggest that the Ras pathway in pancreatic cancer is complicated and non-linear. This knowledge is relevant given the current clinical evaluation of agents which target the Ras pathway in human cancers.

Acknowledgments

We thank Doug Heilman for performing the statistical analyses. This work was funded by a research grant provided by Lilly Research Laboratories.

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